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9620759.2

- 4 OCT 1996

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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

Great Britain

4. Title of the invention

RETROVIRUS

5. Name of your agent *(if you have one)*

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RETROVIRUS

This invention relates to new methods of performing gene therapy and to new systems for introducing therapeutic genes into patients.

INTRODUCTION AND PRIOR ART

A number of diseases are amenable to treatment by the delivery of therapeutic nucleic acids to patient's cells. This is referred to as GENE THERAPY (reviewed extensively in Lever and Goodfellow 1995; Culver 1995; Ledley 1995). To achieve gene therapy there must be a method of delivering genes to the patient's cells and additional methods to ensure the effective production of any therapeutic genes. There are two general approaches to achieve gene delivery; these are non-viral delivery and virus-mediated gene delivery. The best characterised virus-mediated gene delivery system uses replication defective retroviruses to stably introduce genes into patients cells. A major disadvantage of non-viral delivery is that the DNA is confined to the initial target cells and is short lived which, for chronic disease, necessitates repeated treatments with the DNA. A major disadvantage of retroviral vectors is that efficient gene transfer is only achieved by transducing cells *ex vivo* and introducing either the transduced cell population back into the patient or grafting in a cell line that is engineered to release retroviral vector particles. These procedures require significant surgical procedure and manipulation of cells. In addition transduction of patients cells with retroviral vector particles is inefficient.

The various known technologies involved in the field of the invention are described in more detail below.

1. The production of retroviral vectors from multiple separate DNA sequences

It is known that the separate expression of the components of a retroviral vector on separate DNA sequences cointroduced into the same cell will yield retroviral particles carrying defective retroviral genomes that carry therapeutic genes (e.g. Reviewed by Miller 1992). This cell is referred to as the PRODUCER CELL. There are two procedures for generating producer cells. In one, the sequences encoding retroviral GAG, POL and

ENV proteins are introduced into the cell and stably integrated into the cell genome; a stable cell line is produced which is referred to as THE PACKAGING CELL LINE. The retroviral vector genome is then introduced into the packaging cell line by transfection or transduction to create a stable cell line that has all of the DNA sequences required to produce a retroviral vector particle. The second approach is to introduce the three different DNA sequences that are required to produce a retroviral vector particle i.e. the *env* coding sequences, the *gag-pol* coding sequence and the defective retroviral genome into the cell at the same time by transient transfection and the procedure is referred to as TRANSIENT TRIPLE TRANSFECTION (Landau & Littman 1992; Pear et al 1993;). The triple transfection procedure procedure has been optimised (Soneoka et al 1995; Finer et al 1994). WO 94/29438 describes the production of producer cells *in vitro* using this multiple DNA transient transfection method and describes the use of these producer cells *in vitro* to transfer retroviral particles to human cells of any lineage that have been removed from a patient. WO 94/19478 describes the use of novel cell lines for producing high titre retroviral stocks following the transient transfection of one or more retroviral plasmids into a packaging cell line. This also describes only the transfer of retroviruses to target cells *in vitro*. The transfer of retroviruses from a producer cell to a target cell *in vitro* is referred to as COCULTIVATION and it is a well established procedure for introducing retroviruses into cells *in vitro*. There is no prior art that describes the creation of producer cells *in situ* in the patient.

2. DNA mediated gene delivery *in vivo*.

The delivery of genes into a variety of different cells in man or animals using naked DNA or DNA associated with a non-viral delivery system has been well described (reviewed by Ledley 1995). The simplest method involves injecting naked DNA into tissues where it is taken up by a proportion of cells and the genes contained in the DNA are expressed to produce proteins in these cells (Dubensky et al 1984; Wolffe et al 1990) .

The DNA may be delivered by biolistics; in this procedure metal particles are coated with DNA and projected at high velocity into cells by a high pressure device (e.g. Yang et al 1990). The DNA may be coupled to chemical agents that optimise uptake into cells e.g polylysine or

to components of viral particles e.g. adenovirus particles or penton protein or to ligands for specific cognate receptors. The DNA may be encapsulated in liposomes or complexed with cationic lipids (e.g.Hyde et al 1993). Irrespective of how the DNA is delivered by these non-viral methods the seminal feature is that there is no transfer of DNA from the originally transfected cells to other cells except possibly by transfer to daughter cells after cell division. Furthermore the introduced gene is not guaranteed to be permanently maintained in the target cells.

3. Retrovirus mediated gene delivery.

The use of defective retrovirus vectors to deliver genes to target cells is well documented (reviewed by Morgan and Anderson 1993). Defective retroviruses are used to transduce cells that have been removed from the body (*EX VIVO* GENE DELIVERY) or they can be delivered to tissues *in situ* (*IN VIVO* GENE DELIVERY). The vectors introduce DNA into a cell and it is stably incorporated into the host cell genome where it is expressed to produce any therapeutic gene contained within it. There is no dissemination of the therapeutic gene because retroviral vector mediated gene transfer is a one step event that affects only the initial target cell. *In vivo* gene delivery is not widely used because gene delivery is inefficient largely because the retroviral particles delivered in this way are rapidly cleared from the site of treatment and there is no extended exposure of the cells to viral particles. For example when retroviral vector particles were injected into the brains of rats that were carrying glial tumours only a very few cells were transduced by the vectors due to the short, 2-4hrs, half life of the retroviral particles (Short et al 1990).

4. Implantation of producer cells in target tissues

It has been reported that a producer cell that has been created *in vitro* can be implanted into a tissue *in situ* (Short et al 1990) . The producer cell releases retroviral vector particles which then transduce neighbouring cells. In this procedure a producer cell is created by the stable transformation of the cell with the DNA sequences specifiying retroviral components *in vitro*. The cell is cultured *in vitro* and then surgically implanted in the patient. The producer cell is foreign and may be short lived due to destruction by the immune system.

Clearly there is a need for improved ways and means for introducing therapeutic genes into patients. Gene therapy would be significantly simplified if stable introduction of DNA into patient cells could be achieved following non-viral DNA delivery and if the effectiveness of non-viral DNA delivery could be improved. The current invention addresses these needs.

THE INVENTION

In one aspect, the invention provides a DNA sequence or set of DNA sequences encoding a replication defective retroviral vector, for converting cells of a patient into producer cells capable of producing the defective retroviral vector, said retroviral vector comprising at least one therapeutically active gene, the DNA sequence or set of DNA sequences being in a form suitable for administering to the patient and capable of being taken up by the cells.

In another aspect, the invention provides a producer cell capable of producing a defective retroviral vector in a retroviral particle, which vector comprises at least one therapeutically active gene, said producer cell being a fresh cell suitable for introduction into a patient and use in gene therapy.

In a further aspect, the invention provides a method of making a producer cell capable of producing a replication defective retroviral vector, said retroviral vector comprising at least one therapeutically active gene which method comprises introducing a DNA sequence or set of DNA sequences encoding the replication defective retroviral vector, into a fresh mammalian cell to give a producer cell suitable for use in gene therapy.

Preferably, the DNA sequence or set of sequences encoding the replication defective retroviral vector also encode packaging components for production of retroviral vector particles by the producer cells.

As will be explained below in more detail, conversion of the fresh mammalian cells to producer cells may be carried out either *in vivo* or *in vitro*. In the *in vitro* case, the producer cells will be suitable for implanting into a patient, preferably the patient from which the fresh cells were obtained.

It is particularly preferred that the producer cells according to the various aspects of the invention are of a target cell type for which the therapeutically active gene is intended. This avoids the need to introduce any exogenous cells into the target area in a patient.

Further aspects of the invention provide methods of performing gene therapy on a patient, comprising introducing into the patient a producer cell, or a DNA sequence or set of sequences, as described herein.

The term "fresh mammalian cells" as used here refers to mammalian cells which are in their natural state, or as close as possible to their natural state. Cells which have been extensively cultured *in vitro*, including cell lines, are not considered to be fresh cells.

It is an important feature of this invention that the replication defective retroviral vector genome does not encode any of the structural components of retroviral vector particles. The vector thus allows for the insertion of only the therapeutically active gene or genes into the target cell genome. This avoids problems associated with expression of viral components in the target cells, such as undesired immune responses to those components. For example, cytotoxic T cell responses directed against the products of other foreign genes will be avoided.

Thus a set of DNA sequences according to the invention for producing retroviral vector particles having a replication defective retroviral genome, will comprise:

(i) a DNA construct encoding the vector genome, comprising one or more therapeutically active genes plus the remaining components essential for function such as primer binding site, integration sites, packaging signal.

(ii) a DNA construct or constructs encoding *gag-pol* and *env*, preferably as separate constructs.

As will be evident to those skilled in the art, safety considerations to avoid generation of replication competent virus through recombination apply and will be taken into consideration in construction of (i) and (ii) above.

The various DNA constructs described above for use in preparing producer cells according to the invention may be present on separate expression vectors, or they may be present on a single expression vector provided that the vector genome is encoded in a

separate transcription unit. The expression vector or vectors will usually be plasmids.

The invention thus comprises the novel combination of two technologies to produce a method of delivering genes directly to patient/animal tissues such that a long term expression of therapeutic products will be achieved. The essence of the invention is that firstly, using NON-VIRAL DNA DELIVERY, combinations of DNA sequences are introduced into the patients cells. The preferred procedure would not require any removal of cells from the patient. The methods described can however be applied to tissues/organs that are removed from the body and then reimplanted. The combination of DNA sequences is such that when expressed they specify the production of a replication defective retroviral vector genome and the production of the protein components that are required to package that genome to produce retroviral vector particles. The retroviral vector particles are released and then by the process of VIRUS-MEDIATED GENE DELIVERY they attach to and enter additional cells and consequently deliver the defective retroviral genome into the cell where it is copied by the particle associated reverse transcriptase and becomes integrated into the genome of those cells. The cell that originally receives the combination of DNA molecules continues to secrete retroviral vector particles for as long as the cell survives or the DNA persists. This creates an extended opportunity for the retroviral vector particles to transduce cells. The retroviral vector particles contain a therapeutic gene which is expressed in the transduced cells. The invention is therefore to establish a retroviral vector producer cell in the target tissue by the direct delivery of appropriate combinations of DNA sequences. The novelty in this application therefore relates to the combination of two technologies, non-viral DNA delivery and virus mediated DNA delivery, which have hitherto been perceived to be separate and distinct approaches to achieving gene therapy.

Details

DNA is delivered to the cells by any appropriate method including injection, biolistic delivery, carrier mediated delivery. Multiple different DNA sequences on separate molecules or a single molecule carrying multiple different sequences are delivered to human/animal cells. These DNA sequences encode the components of a retroviral vector for

example the HIT system (Soneoka et al 1995) and the kat system (Finer et al 1994). The DNA sequences encode a retroviral Env protein, a retroviral Gag-Pol protein and a replication defective retroviral genome that is engineered to contain one or more therapeutic genes. Additional sequences may also be included. For example a suicide gene such as HSV Tk might be included on one or all molecules to enable transfected and transduced cells to be destroyed by treatment with drugs such as acyclovir (Plautz et al 1991). The combination of DNA sequences are referred to as THE VECTOR PRODUCTION SYSTEM (VPS). The VPS need not be restricted to the three plasmid systems such as HIT and kat but can comprise any retroviral vector system. For example the *env* gene can be engineered to specify an envelope protein that targets retroviral vector particles to a specific cell type, the vector genome can be engineered to contain gene expression signals that confer special properties on the vector e.g. tissue specific expression, regulated expression and the *gag-pol* gene can be engineered to influence infection and integration for example to deliver DNA into the genome of non-dividing cells or to target DNA to a specific site in the chromosome. The cell that receives the VPS is referred to as an *IN SITU* RETROVIRAL FACTORY (ISRF), it is essentially a retroviral vector producer cell created from one of the patient's own cells. The ISRF produces retroviral particles that are released from the cell for as long as the VPS persists, this may be of the order of weeks to months or exceptionally years (Wolff et al 1992). The defective retroviral particles transduce neighbouring cells, referred to as the TARGET CELL POPULATION (TCP), and deliver the therapeutic gene to those cells as a stably integrated provirus. The TCPs do not produce further virus. The ISRF also expresses the therapeutic gene from the VPS. This novel combination of non-viral DNA delivery and virus-mediated gene delivery allows the dissemination of a therapeutic gene throughout a population of patient's cells.

The invention has a number of advantages which relate to the generation of ISRFs in patients cells both in the body and in tissues removed from the body

Advantages

i) **In the patient**

1. Increased efficiency of the delivery of retroviral vectors to target cells in the patient because of the local concentration of viral particles.

2. Increased efficiency of the delivery of retroviral vectors because of the extended time period of exposure of cells to viruses. This means that cells at different stages of the cell cycle have the opportunity to cycle into a phase that is optimum for retroviral infection. These cells would not be available as targets in a single dose treatment with retroviral particles.

3. Creation of an ISRF obviates the need to implant a producer cell that has been generated in the laboratory. Such producer cells are different from patients cells and may even be of non-human origin. These cells are rapidly cleared from most sites of implantation in the body and therefore have limited usefulness.

4. The creation of ISRFs dramatically increases the efficiency of non-viral gene therapy methodologies. In these procedures the transient nature of the expression of the therapeutic gene necessitates frequent multiple repeat treatments with DNA. The ISRF will disseminate therapeutic genes to cells that will continue to produce the product for the life-time of the cell. Treatment need therefore be repeated infrequently if at all for some TCPs.

5. Creation of an ISRF obviates the need to surgically remove patient tissues and transduce them with retroviral vectors before reimplantation. This latter procedure does not allow for further dissemination of the therapeutic gene to other cells. It is technically complex and the cells must be subjected to significant manipulation *in vitro* before reimplantation.

6. The creation of ISRFs increases the probability of obtaining therapeutic gene expression in the majority of tumour cells and hence increases the probability of tumour clearance.

7. The ISRF technology has a variety of therapeutic uses. For example but not restricted to:-

i) Cystic fibrosis (CF): The VPS is introduced into lung tissue e.g by liposome mediated DNA delivery. The consequently

established ISRFs spread the appropriate therapeutic gene CFTR (cystic fibrosis transmembrane conductance regulator) throughout the pulmonary tissue. This confers extended relief of the pulmonary symptoms of CF.

ii) Parkinsons disease: The VPS is introduced into cells in the brain by biolistic delivery over a small surgically exposed area. The consequently established ISRFs deliver a retroviral vector to specific cells e.g. glial cells or astrocytes to deliver relevant therapeutic genes e.g. Tyrosine hydroxylase and dopa decarboxylase.

iii) Alzheimers disease: The VPS is introduced into cells in the brain. The consequently established ISRFs deliver the appropriate therapeutic gene e.g Nerve growth factor.

iv) Tumours: The VPS is delivered to the tumour. The consequently established ISRFs deliver a retroviral vector to surrounding tumour cells to deliver relevant therapeutic genes e.g. HSV thymidine kinase (Tk) or foreign histocompatibility antigens.

ii) In patients tissues *ex vivo*

Advantages i) 1-4 also apply to *ex vivo* applications of ISRFs

1. Direct transduction of a patient's cells *ex vivo* with retroviral vector particles requires the large scale production of high titre retroviral vectors and is often not very efficient requiring prolonged cell culture and genetic selection of transduced cells. An alternative approach is the cocultivation of patients cells with a producer cell line that has previously been created *in vitro*. This may necessitate the separation of the target cells from the producer cell before reimplantation of patients cells. The present invention describes a method to convert a patient's cell directly into a producer cell. The retroviral vector particles are then transferred from the producer cell, now referred to as an ISRF, to other patient cells and the organ/tissue/cells can be reimplanted with minimal manipulation. the creation of ISRFs therefore obviates the need for cocultivation with non-patient cells or treatment of cells with retroviral vector particles in any *ex vivo* method of gene therapy

The invention will now be further described in the examples which follow.

EXAMPLES

Example 1.

Construction of an ISRF in a Hela cell monolayer

Hela cells are plated into 60cm dishes and allowed to grow to 80% confluence. Plasmid DNA comprising pHIT456, pHIT111 and pHIT60 is prepared for transfer into Hela cells by standard calcium phosphate precipitation and introduced into cells using the extended overlay method as described in detail in Soneoka et al 1995. pHIT456 contains the amphotropic retroviral envelope gene that allows infection of Hela cells, pHIT111 is a defective retroviral genome that contains the *lac*Z gene and pHIT60 contains the MLV *gag-pol* gene. The coexpression of these plasmids results in the production of retroviral vector particles that can transduce target cells with the *lacZ* gene. This is referred to as configuration A. Briefly, 10μg of each plasmid is coprecipitated with calcium phosphate and the resulting precipitate is placed on the Hela cell monolayer. After 24 hrs the medium is removed and replaced with fresh medium. Replicate dishes are taken at 24hr intervals and the cells are fixed and stained with X-gal to detect the expression of β-galactosidase (Sanes et al 1986). In a control experiment 10μg of plasmid pKV469 is used in place of the retroviral vector plasmid pHIT111. pKV469 is a simple eukaryotic cell expression vector that expresses the *lac*Z gene via the CMV-IE promoter. In this three plasmid configuration no retroviral vector particles are produced. This is referred to as configuration B.

Cells that are expressing β-galactosidase are stained blue with X-gal. After 24hrs β-galactosidase is expressed in both configurations from the vector plasmid pHIT111 and from pKV469. When the cells are counted a similar number is observed in each configuration. After 48hrs there is an increase in the number of blue cells in both cases. In configuration B this is due to cell division and adjacent pairs (doublets) of blue cells are observed. In configuration A there is also an increase in the number of cells but this comprises both an increase in doublet cells and an increase in single cells and also the appearance of foci of blue cells. The

foci comprise more than two cells which could not result from gene transfer by cell division. The foci appear because virus released from the original cells has infected neighbouring cells. The increase in blue cells in configuration A is more marked after 48 hours with multicellular foci and increased numbers of single cells appearing. This pattern of staining is indicative of one or more rounds of retroviral transduction occurring after the initial transfection of the DNA into the Hela cells. In configuration A, ISRFs are established in the Hela cell monolayer and the *lacZ* gene is disseminated through the target cell population. In configuration B, β-galactosidase expression is resticted to the initially transfected cells and some of their progeny. This experiment establishes that repeated retroviral transduction can occur in a simple homogeneous cell population without the addition of fresh cells as would be the case in a standard cocultivation experiment.

Example 2

Dissemination of the *lacZ* gene throughout the pulmonary tissues of mice

The configuration A and configuration B plasmid sets as above are complexed with cationic liposomes DOTAP or DOTMA/DOPE as described by Alton et al 1993) using 10 to 50μg per plasmid. Liposomes containing DNA are introduced into the lungs of the Edinburgh CF transgenic mouse (Dorin et al 1992) using a jet nebuliser (Alton et al 1993). Mice are sacrificed after 2 days and epithelial cells are harvested by pulmonary lavage. This is repeated for replicate mice at 4 and 14 days. At 14 days lungs are sectioned and sections are stained for the presence of β-galactosidase in pulmonary tissue. In configuaration A the number of blue cells increases to a significantly greater extent than in configuration B and in histological sections foci of blue cells are seen in configuration A but not in configuration B. An ISRF has been established with configuration A and the *lacZ* gene is disseminated through lung tissue.

Example 3

Dissemination of the *lacZ* gene throughout the liver of mice

Mice are subjected to partial hepatectomy. Plasmids in configurations A and B are precipitated by calcium phosphate in the presence of 1μm gold particles. Gold particles are delivered to cells using a biolisitic delivery device. After 2, 4 and 14 days animals are sacrificed and liver sections are stained with X-gal. Foci and scattered blue cells are seen in the liver in configuration A only.

Example 4

Dissemination of the *lacZ* gene throughout the colon of mice

Plasmids in configuration A and B are complexed with cationic liposomes and these are delivered to the colon by instillation. After 2, 4 and 6 days animals are sacrificed and histological sections of the colon are stained with X-gal. Foci and scattered blue cells are seen in colonic eptheliuum.

Example 5

Dissemination of the *lacZ* gene into the brains of mice.

Plasmids in configuration A and B are introduced in the brains of mice through a surgical window in the cranium. DNA is delivered by a biolistic device. Mice are sacrificed after 4 days and 4 weeks. Foci and scattered blue cells are seen with configuration A only.

Example 6

ISRF using human HT1080 cells in culture:

Three-plasmid co-transfections were carried out by calcium-phosphate precipitation as described in Soneoka *et al.* (1995). Plasmid pHIT 60 (MuLV *gag-pol* expression plasmid), pHIT 456 (amphotropic *env* expression plasmid), and pHIT 111 (proviral DNA construct containing the *lacZ* gene) were co-transfected into HT1080 cells in duplicate sets of five 10cm dishes (Figure 1). In the first instance, these cells were maintained

for five days. Every day, one dish from one set was fixed and stained with X-gal, and one dish from the other set was harvested and lysed to measure β-galactosidase activity by a colorimetric assay. As a negative control, HT1080 cells were transfected with pHIT 60, pHIT 123 (ecotropic *env* expression plasmid), and pHIT 111, and the same assays were performed as for the amphotropic producers as described above. Another set of five 10cm dishes was mock-transfected and one dish was harvested everyday to monitor cell growth by counting the number of cells using a hemacytometer. The results obtained are presented in Table 1 and Figure 2. The amphotropic virus-producing HT1080 cells showed an increase in *lacZ*-expressing cells and β-galactosidase activity after one day and the levels were maintained thereafter up to day 5. The increase in both *lacZ*-expressing cells and β-galactosidase activity could not be attributed solely to the increase in cell number, since there was no significant cell growth after day 2 and since the ecotropic virus-producing HT1080 cells showed a reduction in the number of *lacZ*-expressing cells (Table 1 and Figure 2). In these experiments, polybrene was not used to enhance virus transduction and titers obtained without the use of polybrene at the standard time of 48 hours post-transfection (day 1) were relatively low, approximately 10^3 LFU/ml on NIH 3T3 cells for both ecotropic and amphotropic viruses, and also on HT1080 cells with the amphotropic virus. Therefore, it can be concluded that in one 10cm dish containing 5ml of media, up to 5×10^3 transducible particles were in suspension capable of spreading to surrounding HT1080 cells.

The procedure was repeated but the cells were maintained for 14 days. The transfected cells were passaged on days 5 and 10 at a ratio of 1:5. Again, a similar phenomenon was observed with amphotropic virus-producing HT1080 cells, in that β-galactosidase activity of cells increased after day 1 and was maintained thereafter (Table 2). A significant increase in activity was observed at day 12 (Table 2). This increase was most likely due to proliferation of cells harboring the *lacZ* gene, rather than the spread of retroviral vectors, as no infectious virus was being produced at this stage (Table 2). β-galactosidase activity of ecotropic virus-producing HT1080 cells remained at almost baseline level throughout the 14 days, suggesting that the *lacZ* gene transduced by the amphotropic virus was maintained and stably expressed.

No significant cell growth appeared to occur during the course of either experiment, except only after the first day and probably after passage of the cells on days 5 and 10 in the second experiment (Tables 1 and 2). Cells were transfected at approximately 80% confluency, since some toxicity appears to occur from the calcium-phosphate transfection. Recovery of cells from the transfection may explain the growth of cells between days 1 and 2. By day 2, the cells were nearing confluency, hence, the lack of detectable cell growth. Although the nature of MuLV requires that cells be in an actively-dividing state for infection to occur, the low virus titers produced from day 3 onwards (Table 2) suggests that growth of the cells after day 2 was insignificant.

Taken together these data demonstrate that it is possible to use in situ retroviral factories as effective means of spreading retroviral vectors through a population of cells.

Table 1 Spread of retroviral vector in HT1080 retroviral producer cells maintained for 5 days

TYPE OF VIRUS PRODUCED[a]	DAY[b]	# OF BLUE CELLS[c]	β-GAL ACTIVITY[d]	# OF CELLS[e]
Amphotropic	1	4	0.02	N/A[f]
(pHIT60,	2	10.4	0.25	N/A
pHIT456,	3	10.4	0.22	N/A
pHIT111)	4	11.2	0.22	N/A
	5	10.1	0.24	N/A
Ecotropic	1	4.5	0.02	N/A
(pHIT60,	2	1.6	0	N/A
pHIT123	3	1	0	N/A
pHIT111)	4	0.7	0	N/A
	5	0.6	0	N/A
Mock transfected	1	N/A	N/A	3.4×10^6
	2	N/A	N/A	7.3×10^6
	3	N/A	N/A	4.0×10^6
	4	N/A	N/A	5.7×10^6
	5	N/A	N/A	7.0×10^6

[a] Three-plasmid co-transfections were performed as described in Chapter 3.

[b] Day 1 corresponds to 48 hours post-transfection.

[c] Cells were fixed and stained with X-gal. The number of blue cells was counted under x40 magnification and the average of 10 fields was recorded .

[d] 100μg of total protein extract was used and absorbance was measured at OD_{420}.

[e] Total number of cells in a 10cm dish was determined using a hemacytometer.

[f] Not applicable.

Table 2 Spread of retroviral vector in HT1080 retroviral producer cells maintained for 14 days

VIRUS TYPE[a]	DAY[b]	β-GAL ACTIVITY[c]	TITERS[d] NIH 3T3	# OF HT1080	CELLS[e]
Amphotropic	1	0.023	3.4×10^3	3×10^2	N/A[f]
	2	0.036	ND[g]	ND	N/A
	3	0.094	3.8×10^2	53	N/A
	4	0.061	ND	ND	N/A
	5	ND	1	0	N/A
	8	0.053	0	0	N/A
	12	0.355	0	0	N/A
	14	0.310	0	0	N/A
Ecotropic	1	0.014	1.8×10^3	0	N/A
	2	0.033	ND	ND	N/A
	3	0.018	3.8×10^2	0	N/A
	4	0.026	ND	ND	N/A
	5	ND	0	0	N/A
	8	0.008	0	0	N/A
	12	0.014	0	0	N/A
	14	0.074	0	0	N/A
Mock-transfected	1	N/A	N/A		2.7×10^6
	2	N/A	N/A		7.2×10^6
	3	N/A	N/A		7.5×10^6
	4	N/A	N/A		7.4×10^6
	8	N/A	N/A		8.7×10^6
	12	N/A	N/A		4.4×10^6
	14	N/A	N/A		6.2×10^6

a Three-plasmid co-transfections were performed as described in Chapter 3. Amphotropic: pHIT60, pHIT456, pHIT111; Ecotropic: pHIT60, pHIT123, pHIT111.

b Day 1 corresponds to 48 hours post-transfection. Cells were split at a ratio of 1:5 on days 5 and 10.

c 30μg of total protein extract was used and absorbance was measured at OD_{420}.

d Titers were obtained by harvesting supernatant from each 10cm dish, filtering through 0.45μm filters, and adding viral supernatant to either NIH 3T3 cells or HT1080 cells in the absence of polybrene. Cells were X-gal stained 48 hours later and titers were obtained in *lacZ*-forming units per ml (LFU/ml).

e Total number of cells from mock-transfected 10cm dishes was counted using a hemacytometer.

f Not applicable.

g Not done.

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CLAIMS

1. A DNA sequence or set of DNA sequences encoding a replication defective retroviral vector, for converting cells of a patient into producer cells capable of producing the defective retroviral vector, said retroviral vector comprising at least one therapeutically active gene, the DNA sequence or set of DNA sequences being in a form suitable for administering to the patient and capable of being taken up by the cells.

2. A DNA sequence or set of sequences as claimed in claim 1, also encoding packaging components for production of retroviral vector particles by the producer cells.

3. A DNA sequence or set of sequences as claimed in claim 1 or claim 2, for converting cells of the patient which are of a target cell type for the therapeutically active gene.

4. A DNA sequence or set of DNA sequences as claimed in any one of claims 1 to 3, present in one or more plasmids.

5. A producer cell capable of producing a defective retroviral vector in a retroviral vector particle, which vector comprises at least one therapeutically active gene, said producer cell being a fresh cell suitable for introduction into a patient and use in gene therapy.

6. A producer cell as claimed in claim 5, which is of a target cell type for the therapeutically active gene.

7. A method of making a producer cell capable of producing a replication defective retroviral vector, said retroviral vector comprising at least one therapeutically active gene, which method comprises introducing a DNA sequence or set of DNA sequences encoding the replication defective retroviral vector, into a fresh mammalian cell to give a producer cell suitable for use in gene therapy.

8. A method as claimed in claim 7, wherein the DNA sequence or set of sequences also encodes packaging components for the production of retroviral vector particles by the producer cell.

9. A method as claimed in claim 7 or claim 8, wherein the fresh cell is of a target cell type for the therapeutically active gene.

10. A method as claimed in any one of claims 7 to 9, wherein the fresh cell is a patients cell and is converted to a producer cell *in vivo*.

11. A method as claimed in any one of claims 7 to 9, wherein the DNA sequence or set of DNA sequences is introduced into the fresh cell *in vitro*.

12. A method as claimed in claim 11, wherein the fresh cell is from a patient and the resulting producer cell is subsequently reimplanted into the patient.

13. A method of performing gene therapy on a patient, which method comprises introducing into the patient a producer cell according to any one of claims 5 to 7.

14. A method of performing gene therapy on a patient, which method comprises introducing into the patient a DNA sequence or set of DNA sequences according to any one of claims 1 to 4.

Figure 1

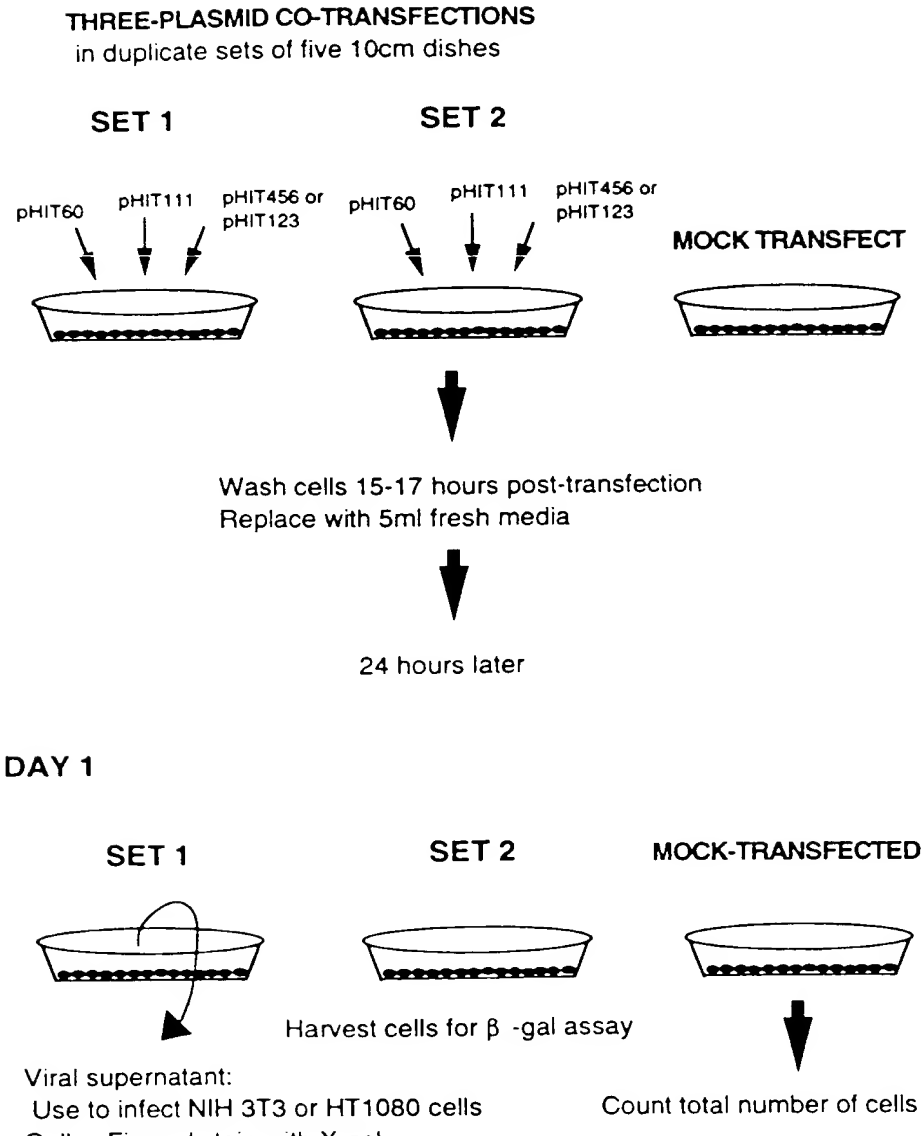
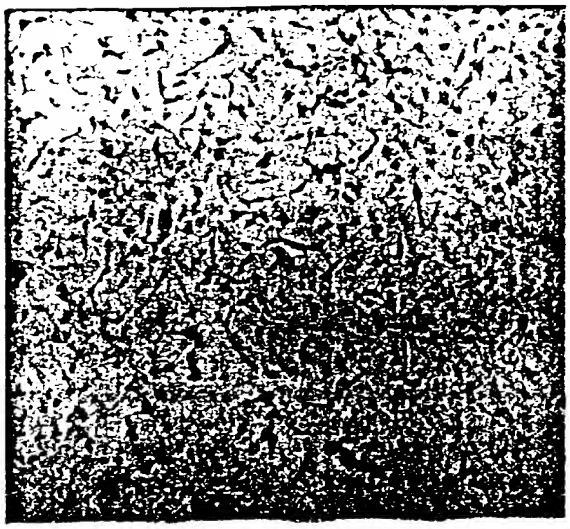

THREE-PLASMID CO-TRANSFECTIONS
in duplicate sets of five 10cm dishes
SET 1
SET 2
pHIT60
pHIT111
pHIT456 or pHIT123
pHIT60
pHIT111
pHIT456 or pHIT123
MOCK TRANSFECT
Wash cells 15-17 hours post-transfection
Replace with 5ml fresh media
24 hours later
DAY 1
SET 1
SET 2
MOCK-TRANSFECTED
Harvest cells for β -gal assay
Viral supernatant:
Use to infect NIH 3T3 or HT1080 cells
Cells: Fix and stain with X-gal
Count total number of cells

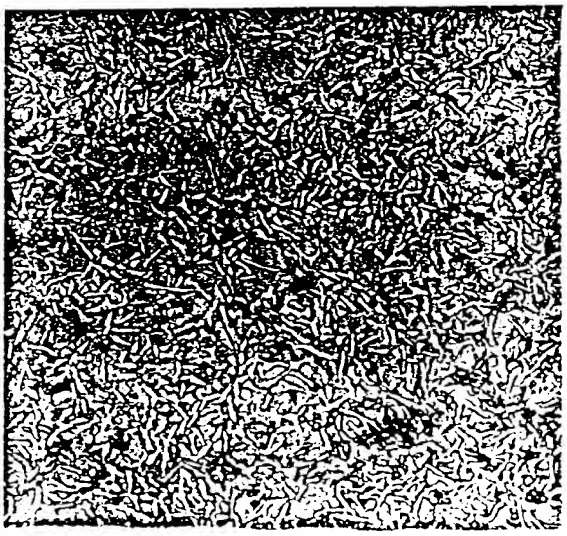
Figure 2

ECOTROPIC

DAY 1



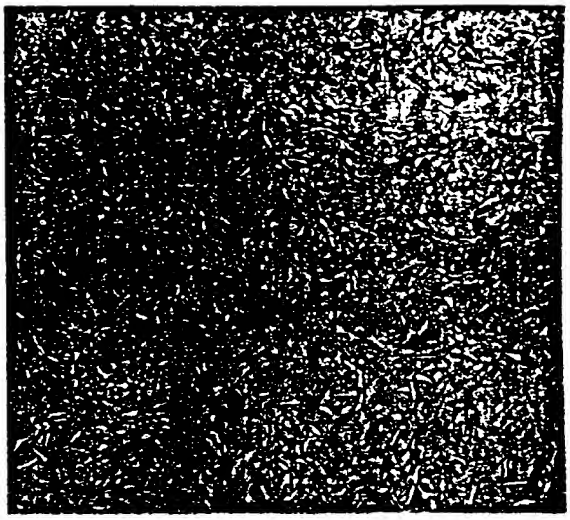
DAY 3



DAY 5

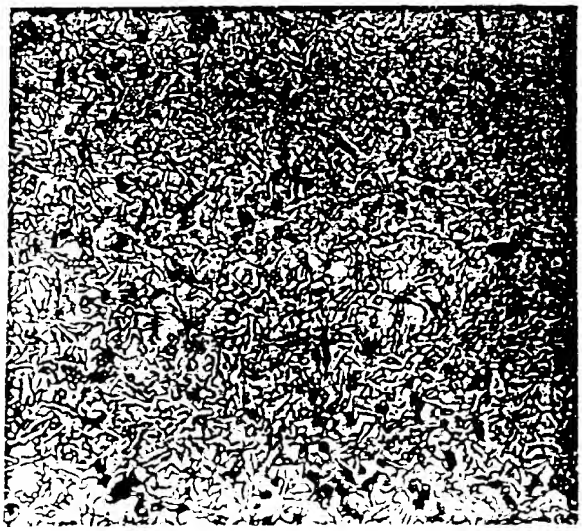
AMPHOTROPIC

DAY 1



DAY 3

DAY 5



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3. Steven Kendall Parker